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Three-dimensional ^{15}N - ^1H - ^1H and ^{15}N - ^{13}C - ^1H nuclear-magnetic resonance studies of HPr a central component of the phosphoenolpyruvate-dependent phosphotransferase system from *Escherichia coli*

Assignment of backbone resonances

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We have performed three-dimensional NMR studies on a central component of the phosphoenolpyruvate-dependent phosphotransferase system of *Escherichia coli*, denoted as HPr. The protein was uniformly enriched with ^{15}N and ^{13}C to overcome spectral overlap. Complete assignments were obtained for the backbone ^1H , ^{15}N and ^{13}C resonances, using three-dimensional heteronuclear ^1H NOE ^1H - ^{15}N multiple-quantum coherence spectroscopy (3D-NOESY-HMQC) and three-dimensional heteronuclear total correlation ^1H - ^{15}N multiple-quantum coherence spectroscopy (3D-TOCSY-HMQC) experiments on ^{15}N -enriched HPr and an additional three-dimensional triple-resonance ^1HN - ^{15}N - $^{13}\text{C}\alpha$ correlation spectroscopy (HNCA) experiment on ^{13}C , ^{15}N -enriched HPr. Many of the sequential backbone ^1H assignments, as derived from two-dimensional NMR studies [Klevit, R. E., Drobný, G. P. & Waygood, E. B. (1986) *Biochemistry* 25, 7760–7769], were corrected. Almost all discrepancies are in the helical regions, leaving the published antiparallel β -sheet topology almost completely intact.

HPr is an 85-amino-acid, water-soluble protein involved in phosphoenolpyruvate-dependent carbohydrate uptake by *Escherichia coli*. It accepts a phosphoryl group from enzyme I and donates it to enzyme II in a chain of phosphorylation/dephosphorylation reactions that results in the phosphorylation and transport of carbohydrates at the expense of phosphoenolpyruvate. In phospho-HPr, the phosphoryl group is attached to the N δ 1 position of the His15 imidazole ring (Weigel et al., 1982a, b; Waygood et al., 1985; Van Dijk et al., 1990). Two conflicting tertiary structures of HPr have been published. Klevit and co-workers used two-dimensional NMR techniques to obtain complete assignments for the backbone and many of the side chain proton resonances, which they used to interpret short-range and long-range NOE (Klevit et al., 1986; Klevit and Drobný, 1986). From these they derived the secondary structure and the tertiary fold of the protein (Klevit and Waygood, 1986). El-Kabbani and co-workers however presented a significantly different structure based on X-ray diffraction studies of HPr crystals grown at acidic pH (El-Kabbani et al., 1987).

This situation prompted us to perform three-dimensional heteronuclear total correlation ^1H - ^{15}N multiple-quantum co-

herence spectroscopy (clean-TOCSY-HMQC) (Marion et al., 1989a) and three-dimensional heteronuclear ^1H NOE ^1H - ^{15}N multiple-quantum coherence spectroscopy (3D-NOESY-HMQC) (Fesik and Zuiderweg, 1988; Marion et al., 1989b) experiments on ^{15}N -enriched HPr. The advantage of these 3D NMR experiments is that spectral overlap in the amide-proton region of the two-dimensional (2D) ^1H -NMR spectra is virtually overcome by spreading these resonances in a third dimension according to the chemical shifts of the backbone ^{15}N nuclei. This greatly simplifies the assignment of the backbone ^1H resonances and also yields assignments of the backbone ^{15}N resonances (Driscoll et al., 1990a, b; Fairbrother et al., 1991). In addition we prepared HPr enriched in both ^{15}N and ^{13}C , and recorded a three-dimensional triple-resonance ^1HN - ^{15}N - $^{13}\text{C}\alpha$ correlation (HNCA) spectrum (Ikura et al., 1990a; Kay et al., 1990), which correlates backbone ^1H , ^{15}N and $^{13}\text{C}\alpha$ resonances. This provides an independent check of the sequential assignments, which will form the basis for further NMR studies on the structural and dynamic properties of HPr.

MATERIALS AND METHODS

Materials

$(^{15}\text{NH}_4)_2\text{SO}_4$ (95% ^{15}N enriched) was obtained from VEB Berlin Chemie. $[^{13}\text{C}_6]\text{glucose}$ (90% ^{13}C enriched) was obtained from Cambridge Isotope Laboratories. All other chemicals were from commercial sources.

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Abbreviations. 2D, two dimensional; 3D, three dimensional; HMQC, heteronuclear multiple-quantum coherence spectroscopy; NOESY, NOE spectroscopy; TOCSY, total correlation spectroscopy; HNCA, three-dimensional triple-resonance ^1HN - ^{15}N - $^{13}\text{C}\alpha$ correlation spectroscopy; rf, radio frequency.

Production of uniformly ^{15}N -enriched HPr

Production of uniformly ^{13}C , ^{15}N -enriched HPr

containing the same components as used for the ^{15}N labeling. Instead of glycerol, $[\text{}^{13}\text{C}_6]\text{glucose}$ (1 g/l) was used as the sole carbon source. Cells were harvested after 24 h of growth in flasks at 37°C , yielding about 15 g cells (wet mass).

Purification of HPr

HPr was purified and prepared for the NMR measurements as described by Van Dijk et al. (1990). HPr solutions were brought to the appropriate pH value by dialyzing against 50 mM KPi , pH 6.5. After freeze-drying, the protein was redissolved in 0.5 ml $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (93:7, by vol.). 70 mg ^{15}N -labeled HPr was obtained from 27 g cells (wet mass). The yield of the ^{13}C , ^{15}N -labeled HPr was 15 mg from 15 g cells (wet mass). ^{15}N -enrichment was over 90%, ^{13}C -enrichment was above 80%, as judged from the intensity of ^{15}N and ^{13}C coupled proton resonances in the NMR spectra. All spectra were run at 30 °C in 50 mM KPi , pH 6.5.

Purity and stability of HPr

The purity of the protein was checked by SDS/PAGE on 15% gels as described by Laemmli (1970), followed by staining with Coomassie brilliant blue. The protein migrated as a

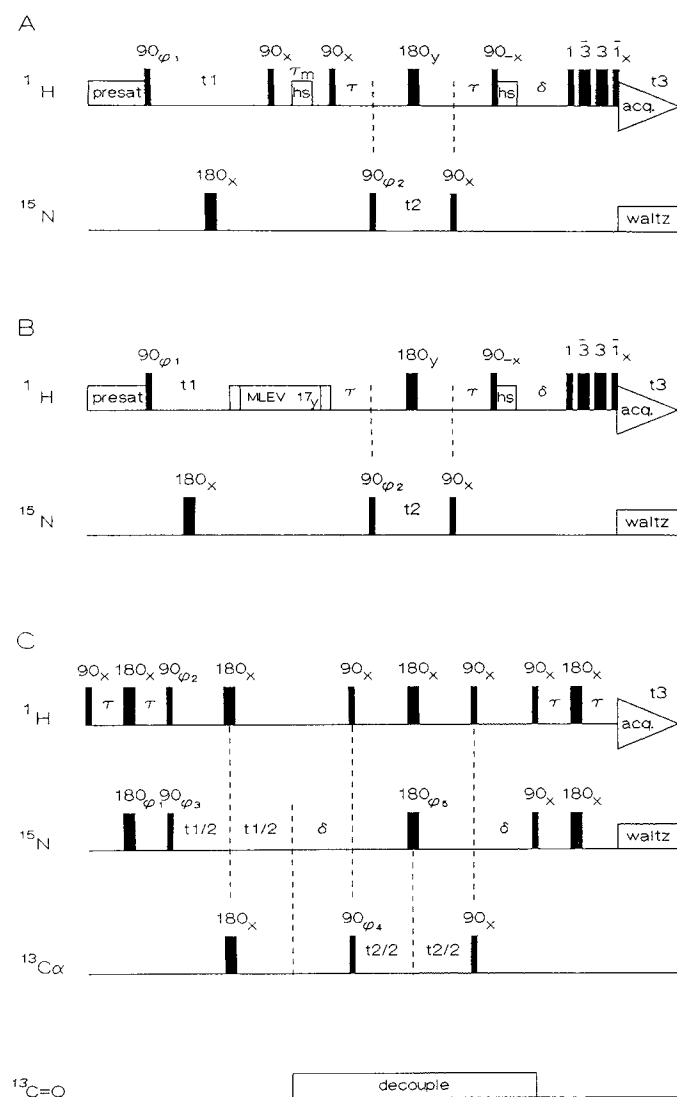


Fig. 1. Pulse sequences. (A) Pulse sequence used for the NOESY-HMQC experiment. The ^1H -carrier frequency was at the water resonance. Time-proportional phase incrementation was used for phases ϕ_1 and ϕ_2 to discriminate between positive and negative ω_1 and ω_2 frequencies, respectively. Maximum t_1 , t_2 and t_3 values were 26, 27 and 154 ms, and spectral widths in the ω_1 , ω_2 and ω_3 domains were 6666, 2000 and 6666 Hz, respectively. Four t_3 transients were recorded for each t_1, t_2 pair. Phases were cycled as follows: ϕ_1 , x,x,x,-x,-x,-x; ϕ_2 , x,-x,-x,-x,-x,x. The delay, τ , was tuned to 5 ms $[(2J_{\text{NH}})^{-1}]$ and a mixing time τ_m of 0.1 s was used. The homospoil pulse (hs) was positioned at the end of the mixing time. During acquisition ^{15}N decoupling was achieved by a broadband Waltz decoupling sequence (waltz2) using an rf field with $\gamma B_1 = 1$ kHz. During the relaxation delay of 1.5 s (presat) the water resonance was irradiated by a weak rf field. Other delays used, $\delta = 20$ ms, $\tau_{\text{hs}} = 10$ ms, $\delta_{1,3,3,1} = 222$ μs . Total duration of the experiment was about 85 h. (B) Pulse sequence used for the clean-TOCSY-HMQC experiment. During the 56-ms mixing time a MLEV17 pulse train was applied (Bax and Davis, 1985) using an rf field with γB_1 of 10.9 kHz and with 46- μs delays bracketing the π pulses in the MLEV17 sequence (Griesinger et al., 1988). 2-ms trim pulses were applied immediately before and after the MLEV17 sequence (Bax and Davis, 1985). Further details were as described for the NOESY-HMQC experiment. (C) Pulse sequence used for the HNCA experiment. Maximum t_1 , t_2 and t_3 values were 19, 8 and 77 ms, respectively and spectral widths in the ω_1 (^{15}N), ω_2 (^{13}C), and ω_3 (^1H) domains were 2000, 4000 and 6666 Hz, respectively. The following delays were used, $\tau = 2.25$ ms and $\delta = 22$ ms. 16 t_3 transients were recorded for each t_1, t_2 pair. Phases were cycled as follows: ϕ_1 , x,-x,-x; ϕ_2 , y,-y,-y; ϕ_3 , x; ϕ_4 , 2(x),2(-x); ϕ_5 , 4(x),4(y),4(-x),4(-y); acq, x,2(-x),x,-x,2(x),-x. Time-proportional phase incrementation was applied to phases ϕ_3 and ϕ_4 to discriminate between positive and negative frequencies in the t_1 and t_2 domains. The water resonance was suppressed by presaturation with a weak rf field. Total duration of the experiment was 36 h (spectra of comparable quality can be obtained in less than 20 h, using a maximum t_1 value of ≈ 10 ms). Following Marion and Bax (1989), the t_1 and t_2 data points were sampled at times equal to integer numbers multiplied by the increment used, enabling the data points corresponding to $t_1 = 0$ and $t_2 = 0$ to be reconstructed by linear prediction methods, thus avoiding the need for a frequency-dependent phase correction in these domains. In the t_2 domain, linear prediction was also used to double the number of data points to 128, resulting in a significant improvement of the resolution in this domain.

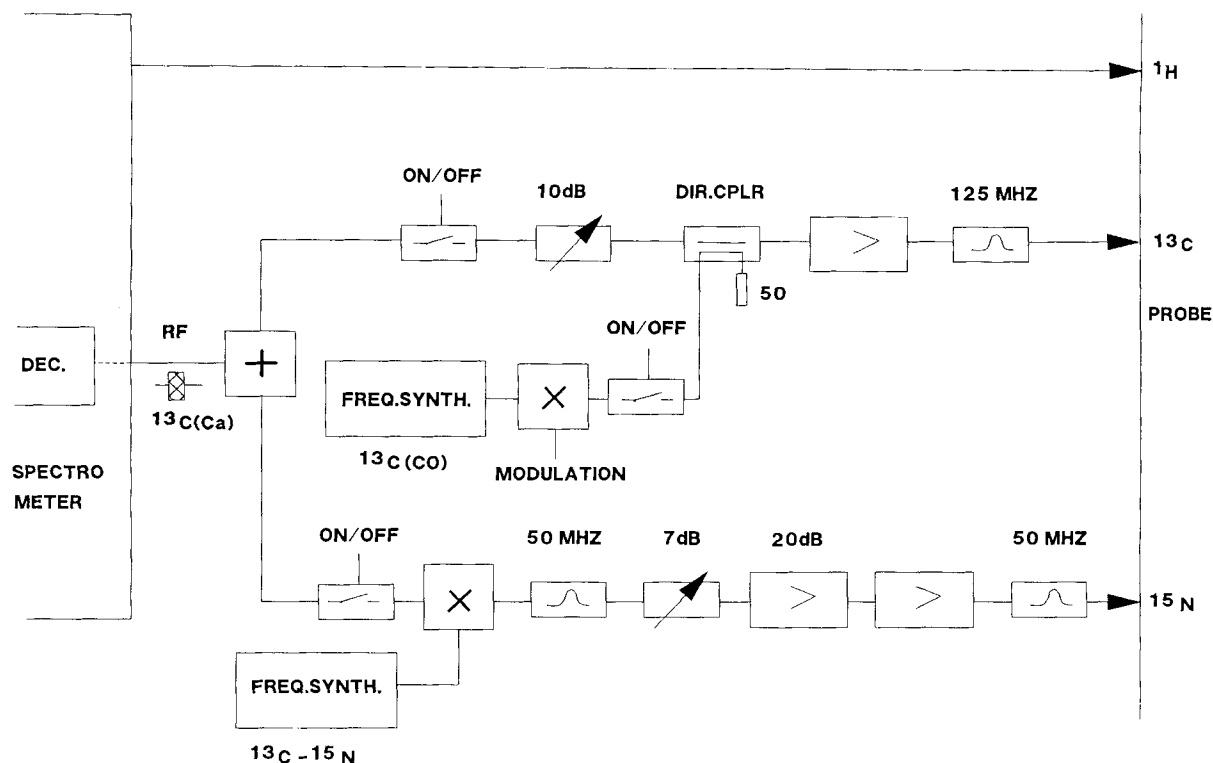


Fig. 2. Block diagram of the hardware modifications of the decoupler channel (DEC) needed for the HNCA experiment. Two extra frequency synthesizers (FREQ.SYNTH.) were used to deliver the carbonyl ^{13}C frequency (PTS 160) and the difference frequency ($^{13}\text{C} - ^{15}\text{N}$) (PTS 500). The power splitter (+), double balanced mixers (\times) and the directional coupler (DIR.CPLR) were purchased from Mini-circuits Corp. The bandpass filters are from Texscan Corp. The two power amplifiers ($>$) (ENI 525 and ENI 5100) are part of the standard spectrometer. The 20 db amplifier (HP461A) delivers sufficient power to drive the ^{15}N power amplifier (ENI 5100). The two rf switches (home built) are driven by two computer-controlled lines and deliver an attenuation of 70 db.

single, homogeneous band and showed no loss of activity during the NMR measurements. This was checked by determining the HPr activity prior to and after the measurements in the HPr mutant complementation assay, described by Kundig and Roseman (1971) and modified as described by Van Dijk et al. (1990). HPr is known to be susceptible to deamidation of glutamine and asparagine residues (Anderson et al., 1971; Waygood et al., 1985). We found no indication for this in our samples, as judged by the constant intensity of the characteristic cross peaks for the side chain amino groups of Gln and Asn.

Determination of the HPr concentration

The HPr concentration was determined by the pyruvate-burst method as described by Robillard and Blaauw (1987) and slightly modified as described by Van Dijk et al. (1990). The protein concentration in the ^{15}N -enriched HPr NMR sample was ≈ 6 mM and for the ^{13}C , ^{15}N -enriched HPr NMR sample the protein concentration was ≈ 2 mM.

^{15}N - ^1H - ^1H NMR spectroscopy

All NMR experiments were performed on a Varian VXR 500 MHz NMR spectrometer.

The NOESY-HMQC (Fesik and Zuiderweg, 1988; Marion et al., 1989b) and the clean-TOCSY-HMQC (Marion et al., 1989a) pulse sequences are shown in Fig. 1A and B. The strong water resonance was suppressed by presaturation with a weak radio-frequency (rf) field and by inserting a

z-filter (Sørensen et al., 1984), including a z-gradient pulse, before the frequency-selective 1331-excitation pulse (Hore, 1983).

^{15}N - ^{13}C - ^1H NMR spectroscopy

The pulse sequence for the HNCA experiment (Kay et al., 1990) is shown in Fig. 1C. A 5-mm triple-resonance probe was obtained from Varian. The decoupler channel of the Varian VXR-500 spectrometer was modified in order to apply the ^{15}N and ^{13}C pulses (Fig. 2). The scheme includes a fourth channel, which is used to apply a weak rf field at the carbonyl ^{13}C frequency, derived from a separate synthesizer. The ^1H channel is operating in the standard way and no modifications are necessary. The decoupler channel, broadband by nature, delivers the ^{13}C frequency (rf) and is set to a frequency in the $^{13}\text{C}\alpha$ region. The rf pulses are fed into a power splitter and serve as an input for both the ^{13}C and ^{15}N channels. The $^{13}\text{C}\alpha$ signal from the first output of the power splitter is combined with the carbonyl ^{13}C signal via a software-controlled rf gate. A square wave modulation of 90 Hz results in an adequate decoupling of the CO region. Both the $\text{C}\alpha$ and carbonyl signals are fed through the final power amplifier (ENI 525) into the probe. On its second output the power splitter also delivers a pulse with the $\text{C}\alpha$ frequency. When a ^{15}N pulse is needed, the rf switch passes this pulse to a balanced mixer. The second input of the mixer is obtained from a synthesizer which delivers a frequency that is the difference between the $^{13}\text{C}\alpha$ and the desired ^{15}N frequency. The lower sideband hence comprises the desired ^{15}N frequency. This ^{15}N signal is fed to the ^{15}N

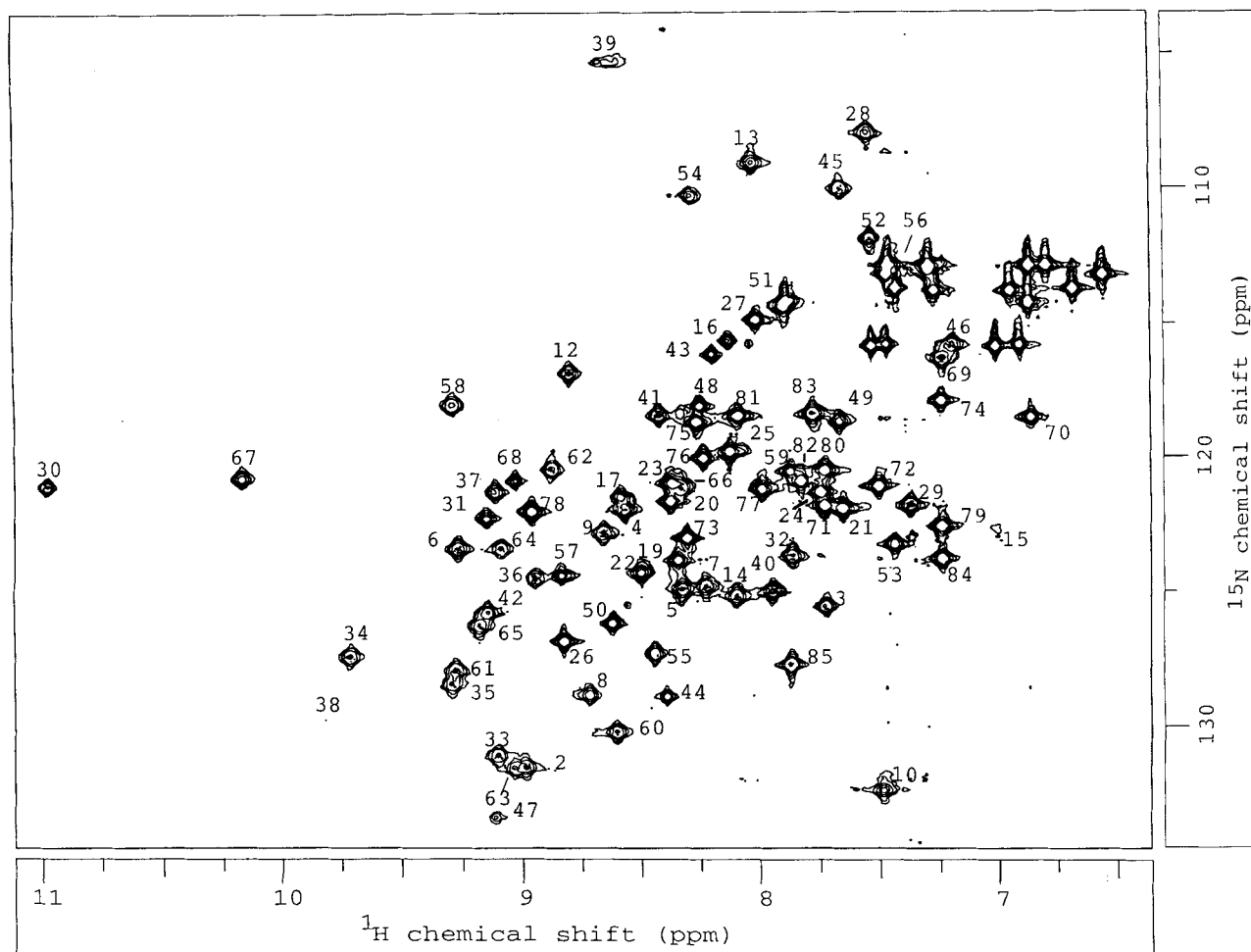


Fig. 3. The ^{15}N -HMQC spectrum of ^{15}N -enriched HPr at 30°C , in 50 mM KP_i, pH 6.5. The contours represent the resonances of all the protons covalently linked to ^{15}N nuclei with the exception of the amide proton of Met1 (see text). The horizontal axis is the directly observed proton chemical shift. The vertical axis is the indirectly observed ^{15}N chemical shift. The numbers represent the residue number in the amino acid sequence that follow from our sequential assignments.

input of the triple-resonance probe via appropriate gating, filtering and amplification. With this scheme frequencies, phases and strength of the decoupler pulses can be programmed in the usual way, even when applied simultaneously with a transmitter ^1H pulse. Simultaneous ^{15}N and ^{13}C pulses can be applied as well, provided that they have the same duration, but their phases and strength cannot be varied independently.

Recording, data storage, processing, visualisation and analysis: the SNARF program

Multi-dimensional NMR experiments require flexible software for processing, visualizing and analyzing the large data sets. For all these tasks we used the program SNARF, written by one of us (Frans van Hoesel). Typically, a 3D NMR spectrum is recorded as a series of 2D spectra. Each 2D spectrum, when completed, is copied via an Ethernet link from the local NMR disk to a separate directory on one of the disks mounted on a remote computer, usually a Convex C1. This is controlled by a macro (written in Varian's Magical language), which also increments the delay corresponding to the third evolution time and starts the next 2D experiment. SNARF is used on the remote computer to process the spectra, combine

them in a truly 3D dataset (usually after Fourier transformation in one or two of the time domains) and finish the processing in the remaining domain(s). The same program was used for all data manipulations described in the following sections.

Assignment procedure

The NOESY-HMQC experiment correlates the ^{15}N and ^1H (ω_2, ω_3) frequencies of each of the backbone and side chain (Gln, Asn and Arg) ^{15}N -HN moieties with those of protons closer than ≈ 0.5 nm via the NOE. The TOCSY-HMQC experiment correlates the frequencies of the same ^{15}N -HN pairs with those of protons that are J coupled, directly or indirectly, with the backbone amide proton. Traces, ω_1 , through the NOESY-HMQC spectrum, at the (ω_2, ω_3) frequencies of all identified ^{15}N -HN spin systems, are compared with all ω_1 traces through the TOCSY-HMQC spectrum. If at least two frequencies in the NOESY and TOCSY traces are the same, these traces, numbered arbitrarily, are regarded as correlated and the corresponding element of a correlation matrix is filled in. The type of correlation is encoded in the correlation matrix using a numbering system, which reflects the number of frequency matches and also the type of protons involved in the

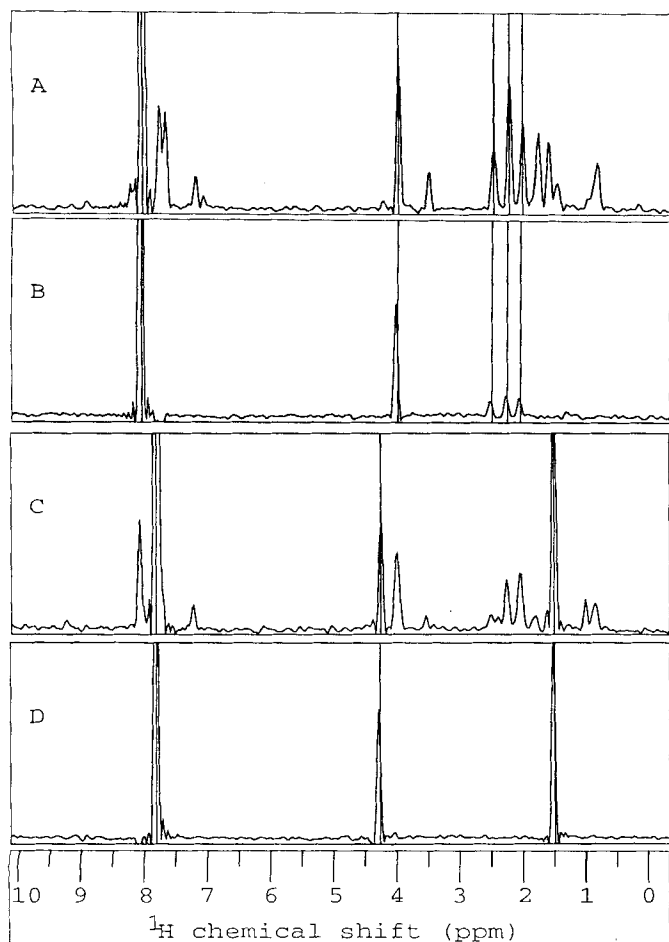


Fig. 4. ω_1 traces through the NOESY-HMQC (A + C) and clean-TOCSY-HMQC (B + D) spectra of HPr at two different (ω_2, ω_3) frequency pairs, corresponding to the ^{15}N -HN spin pairs of Met81 (A + B) and Ala82 (C + D). The vertical bars indicate the spin systems as deduced from the clean-TOCSY-HMQC experiment. Note the small shifts in frequency between corresponding resonances in the NOESY and TOCSY traces, which are due to a small ($\approx 0.5^\circ\text{C}$) difference in the temperature at which the spectra were recorded.

match. The simultaneous occurrence of, e.g. amide and H α frequencies in NOESY and TOCSY ω_1 traces is thus remembered. The sequential assignment now reduces to a re-shuffling of this correlation matrix in such a way that strongly correlated ω_1 traces become neighbours, giving the matrix a characteristic diagonal appearance.

RESULTS

Fig. 3 shows a ^{15}N -HMQC spectrum of ^{15}N -enriched HPr. Since Met1 and Pro11 and Pro18 will not give rise to cross peaks in this spectrum, we expect 82 cross peaks correlating the resonances of the amide protons with those of their attached backbone nitrogens. The number associated with each peak represents the residue number in the amino acid sequence which follows from our sequential assignments. All expected cross peaks could indeed be identified. Note the low intensities of the peaks for His15 and Asn38, which are probably due to rapid exchange of these amide protons with H_2O protons under these conditions. Of these, the Asn38 cross peak is stronger in a spectrum recorded at 20°C (not shown). The

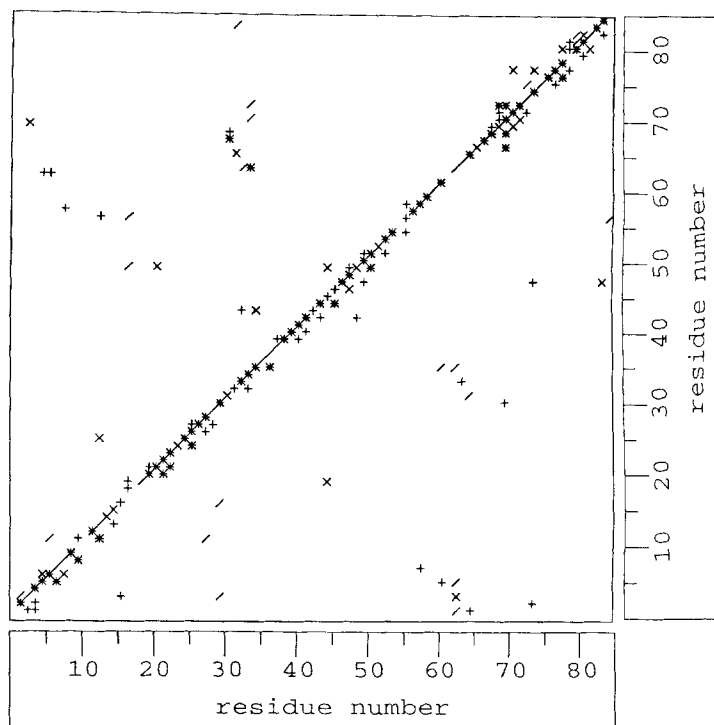


Fig. 5. Correlation matrix described in the text. A symbol at position (i, j) in these matrices stands for the observation of at least two NOE between the amide proton of residue j [$\text{HN}(j)$, vertical axis] with protons that belong to the spin system of residue i (horizontal axis), as deduced from the TOCSY-HMQC experiment. Correlations are encoded by four different symbols. (*) strong NOE were observed in the NOESY trace of $\text{HN}(j)$ at all frequencies that define spin system i ; (x) NOE were observed in the NOESY trace of $\text{HN}(j)$ at two or more frequencies of spin system i , including strong NOE at the $\text{HN}(i)$ and $\text{H}\alpha(i)$ frequencies; (+) weak NOE were observed in the NOESY trace of $\text{HN}(j)$ at all frequencies that define spin system i ; (/) NOE were observed in the NOESY trace of $\text{HN}(j)$ at two or more frequencies of spin system i , including the $\text{H}\alpha(i)$ (strong) but not the $\text{HN}(i)$ frequency. Numbers 1–82 correspond to the ^{15}N -HN cross peaks in the ^{15}N -HMQC spectrum, shown in Fig. 3. They represent the residue numbers in the amino acid sequence.

side chain NH_2 groups of the two Asn and six Gln residues give rise to 16 additional peaks which cluster between 8.0 and 6.5 ppm (^1H) and 120 and 110 ppm (^{15}N). These cross peaks lack numbers. Their assignment will be treated at a later date.

Assignment procedure

The assignment procedure is based on the analysis of a NOESY-HMQC experiment together with a TOCSY-HMQC experiment on ^{15}N -enriched HPr. Fig. 4 illustrates how these two data sets are used to arrive at the sequential assignments. A nitrogen and proton (ω_2, ω_3) frequency pair is selected corresponding to one of the amide proton cross peaks in the HMQC spectrum in Fig. 3. All proton resonances in the third dimension (ω_1) having the same ω_2 and ω_3 frequencies in the NOESY-HMQC and TOCSY-HMQC experiment are then plotted as traces. Fig. 4A and B compare the NOESY-HMQC (A) and TOCSY-HMQC (B) traces for the HMQC cross peak of the amide proton for residue 81. The resonances in the TOCSY trace (Fig. 4B) identify the protons of the spin system of Met81. As expected, we find the same resonances in the NOESY trace (Fig. 4A) for residue 81 in addition to other

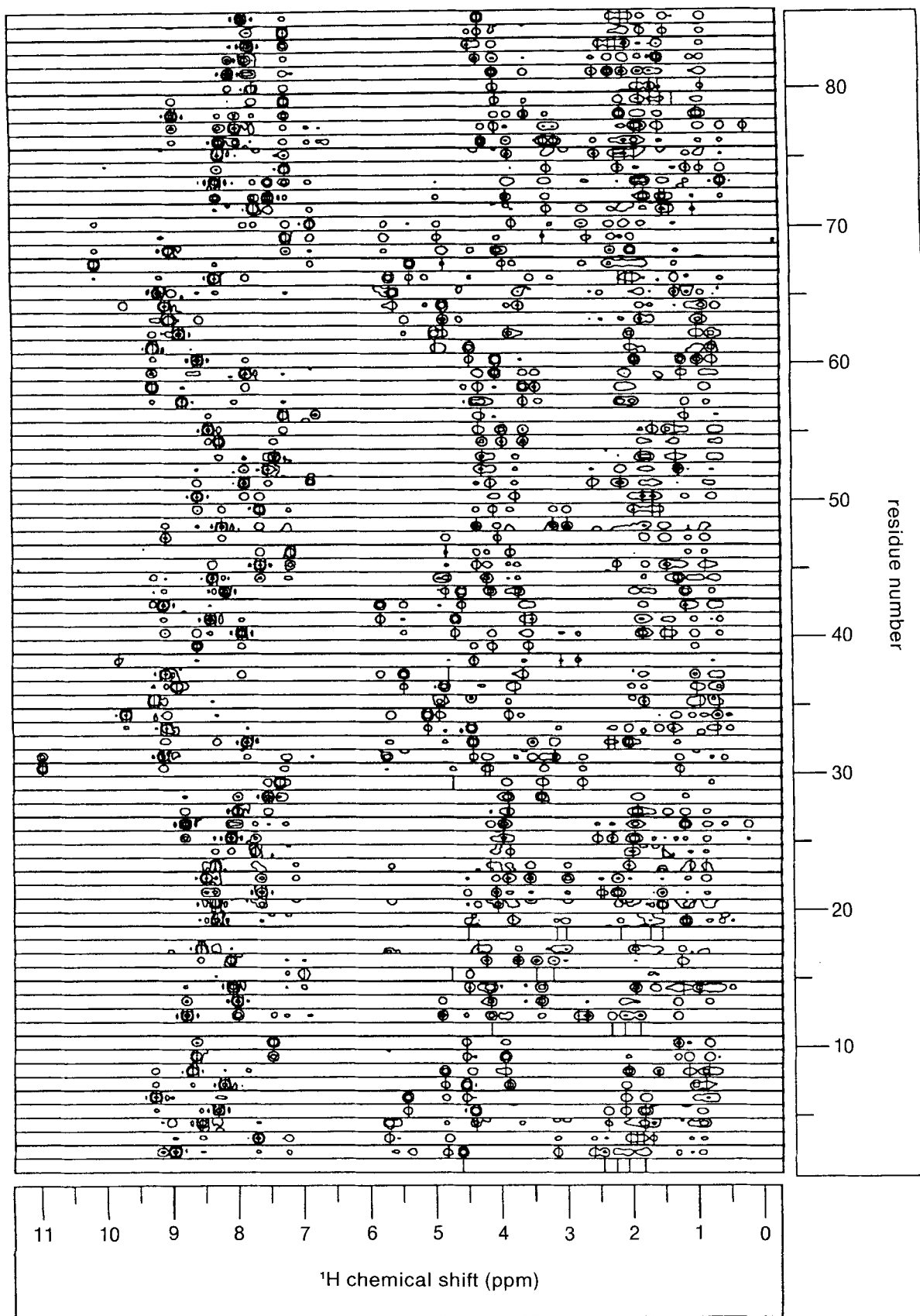


Fig. 6. Contour plots of slices taken from the NOESY-HMQC spectrum that correspond to all observed backbone ^{15}N -HN spin pairs. Each slice shows the full ω_1 (^1H) frequency domain and a narrow band around the ω_2 (^{15}N) frequency of the corresponding ^{15}N -HN cross peak in the HMQC spectrum. They are sorted with respect to the position of the ^{15}N -HN spin pair in the amino acid sequence. Resonances that were also observed in the TOCSY-HMQC spectrum are indicated by vertical bars. The resonance frequencies of the spin systems of Met1, Pro11, Pro18 and His15 were observed as NOE with the $\text{H}\alpha$ protons in a ^{13}C -NOESY-HMQC experiment, which were identified using the $^{13}\text{C}\alpha$ frequencies derived from the HNCA spectrum.

resonances from protons of nearby residues. But we also find these same resonances in Fig. 4C, the NOESY trace corresponding to Ala82. Statistics on known protein structures (Billeter et al., 1982) show that the simultaneous occurrence of NOE between a backbone amide proton of a certain residue and the backbone amide and H α protons of a different residue identifies these residues with high probability as neighbours in the amino acid sequence of the protein. This is the basis of the sequential assignment procedure proposed by Wüthrich et al. (1982) and our own strategy.

In the first stage of the assignment procedure we searched for correlations between NOESY and TOCSY ω_1 traces of the type shown in Fig. 4. Correlations that were based on at least two frequency matches in the corresponding NOESY and TOCSY traces were collected in a correlation matrix. They were ultimately sorted using the following criteria: (a) strongly correlated traces most likely involve neighbouring residues, (b) correlations that involve an intense HN-H α cross peak are positioned preferably on one side of the diagonal, such that the correlation corresponds to a HN-H α ($i, i-1$) contact; in contrast, intense HN-HN contacts can be bidirectional, (c) the nature of the spin system as seen in the TOCSY traces must not be in disagreement with the type of amino acid at that particular position in the amino acid sequence.

The result of this sorting process is shown in Fig. 5, where the numbering corresponds to the position of the ^{15}N -HN spin pair in the amino acid sequence. This yielded complete sequential assignments of the backbone protons and nitrogen atoms, which are collected in Table 1. Excluding connectivities involving Met1, Pro11 and Pro18, three open spaces are left in this correlation matrix due to overlap between H α protons of neighbouring residues. Residues 55 and 56, and residues 79 and 80 show HN-HN connectivities, while the connection between residues 62 and 63 is based on side chain cross peaks.

Since our assignments are not consistent with those reported by Klevit et al. (1986) we present all of the experimental evidence in Fig. 6, used to make these assignments. Fig. 6 shows contour plots of slices taken from the NOESY-HMQC spectrum at the ^1H (ω_3) frequency corresponding to each backbone amide proton. Each slice covers the full ^1H spectral width (ω_1) and a narrow band around the ^{15}N (ω_2) frequency of the ^{15}N -HN spin pair. The slices are stacked to correspond to their position in the amino acid sequence. Resonances that were also observed in the TOCSY-HMQC spectrum are indicated by vertical bars. Parts of the spin systems of Met1, Pro11, Pro18, and His15, were derived from a ^{13}C -NOESY-HMQC (Ikura et al., 1990b; Zuiderweg et al., 1990) spectrum of HPr to be described in a following paper. Helices, apparent from uninterrupted stretches of strong HN-HN($i, i-1$) and weak HN-H α ($i, i-1$) cross peaks, can be seen to run from residues 20–27, 47–52 and 71–85. β -Strands, apparent from stretches of strong HN-H α ($i, i-1$) and weak HN-HN($i, i-1$) cross peaks, run from residues 1–7, 31–37, 40–44 and 60–67. Fig. 8 summarizes cross-strand NOE that were identified in our 3D-NOESY-HMQC spectra and lead to the indicated antiparallel arrangement of the four β -strands.

We recorded a triple-resonance HNCA spectrum as an independent check of the backbone sequential assignments. It provides the $^{13}\text{C}\alpha$ resonance assignments as well. This experiment was originally devised to correlate intra-residue ^{15}N , ^1H and $^{13}\text{C}\alpha$ resonances, but it also shows inter-residue connectivities between ^{15}N -HN moieties of certain residues with the $^{13}\text{C}\alpha$ of preceding residues, by direct transfer via the $^2J_{\text{NC}\alpha}$ coupling, which can be as large as 7 Hz (Kay et al., 1990). Fig. 7 shows this HNCA spectrum, again as contour

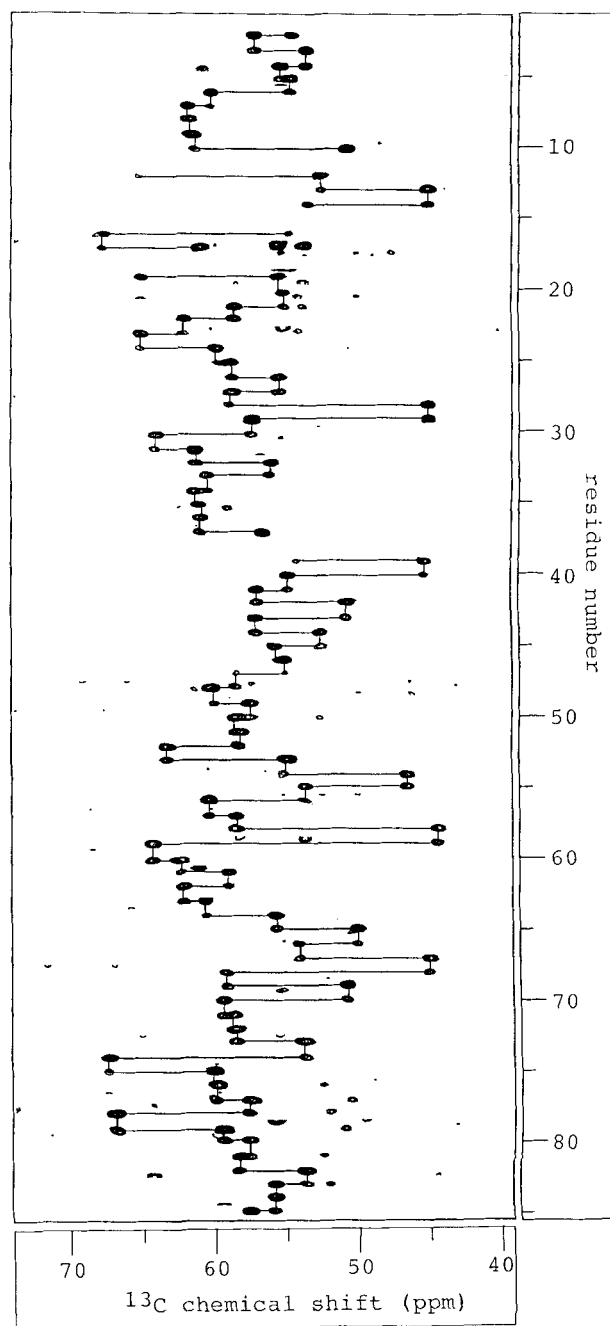


Fig. 7. HNCA spectrum of ^{13}C , ^{15}N -enriched HPr at 30°C in 50 mM KP_i, pH 6.5, as contour plots of 2D slices taken from the 3D spectrum covering the full ^{13}C spectral width and a narrow band around the ω_1 (^{15}N) frequency of the corresponding ^{15}N -HN cross peak. The plots were sorted, like those in Fig. 6, according to the assignment of the corresponding ^{15}N -HN spin pair. Note the typical up-field shifted C α resonances of the glycines, all fairly well in agreement (within ≈ 1.5 ppm) with the value reported for small linear peptides (Hawart and Lilley, 1978).

plots of 2D (ω_1, ω_2) slices taken from the 3D spectrum at the ω_3 frequencies of the amide protons. Each slice covers the full ^{13}C spectral width (ω_2) and a narrow band around the ^{15}N (ω_1) frequency of the ^{15}N -HN spin pair. Again, the slices are sorted according to the position of the residue in the amino acid sequence. At each layer two cross peaks can be identified,

Table 1. Polypeptide backbone ^1H , ^{13}C and ^{15}N chemical shifts for *E. coli* HPr (30°C, 50 mM KP_i, pH 6.5). ^1H chemical shifts are expressed relative to DSS, ^{15}N shifts relative to liquid NH_3 (Live et al., 1984) and ^{13}C shifts relative to hypothetical internal (trimethylsilyl)propionic acid (Bax & Subramanian, 1986).

Residue	Chemical shift				Residue	Chemical shift			
	^{15}N	HN	$^{13}\text{C}\alpha$	H α		^{15}N	HN	$^{13}\text{C}\alpha$	H α
1 Met ^a			54.7	4.60	44 Ala	128.9	8.40	52.6	4.21
2 Phe	131.5	8.99	57.2	4.80	45 Lys	110.0	7.67	55.6	4.35
3 Gln	125.5	7.73	53.6	5.73	46 Ser	115.8	7.20	54.9	4.83
4 Gln	121.9	8.57	55.4	4.40	47 Leu	133.3	9.11	58.4	4.04
5 Glu	124.9	8.33	54.7	5.44	48 Phe	118.1	8.26	60.0	4.36
6 Val	123.4	9.27	60.2	4.54	49 Lys	118.8	7.67	57.4	4.10
7 Thr	124.8	8.23	61.8	4.87	50 Leu	126.2	8.62	58.4	3.76
8 Ile	128.9	8.72	61.7	3.95	51 Gln	114.1	7.91	58.1	4.14
9 Thr	122.8	8.66	61.3	4.54	52 Thr	111.8	7.54	63.1	4.27
10 Ala	132.3	7.49	50.6	4.55	53 Leu	123.3	7.44	54.8	4.27
11 Pro ^a			65.1	4.17	54 Gly	110.3	8.29	46.6	3.97, 3.65
12 Asn	116.9	8.80	52.5	4.91	55 Leu	127.4	8.45	53.6	4.32
13 Gly	109.1	8.03	45.0	4.17, 3.40	56 Thr	112.9	7.31	60.3	4.26
14 Leu	125.2	8.10	53.3	4.49	57 Gln	124.4	8.84	58.4	3.64
15 His ^a	122.6	7.02	54.7	4.76	58 Gly	118.0	9.29	44.5	4.32, 3.46
16 Thr	115.7	8.13	67.6	3.76	59 Thr	120.6	7.88	64.2	4.06
17 Arg	121.5	8.59	60.9	4.36	60 Val	130.2	8.61	62.1	4.45
18 Pro ^a			64.9	4.51	61 Val	127.9	9.28	58.9	4.98
19 Ala	123.9	8.35	55.3	3.84	62 Thr	120.5	8.88	62.0	4.85
20 Ala	121.6	8.38	55.1	4.07	63 Ile	131.5	9.03	60.5	4.86
21 Gln	121.9	7.66	58.4	4.09	64 Ser	123.4	9.09	55.7	5.61
22 Phe	124.4	8.50	61.9	3.91	65 Ala	126.3	9.18	50.1	5.68
23 Val	121.0	8.37	65.0	4.17	66 Glu	121.2	8.34	54.0	5.36
24 Lys	121.3	7.75	59.7	3.88	67 Gly	120.8	10.17	45.1	4.85, 3.95
25 Glu	119.8	8.13	58.7	3.98	68 Glu	120.9	9.03	59.1	4.03
26 Ala	126.8	8.83	55.4	3.97	69 Asp	116.3	7.24	50.7	4.93
27 Lys	114.9	8.02	58.7	3.89	70 Glu	118.5	6.87	59.3	3.79
28 Gly	107.9	7.55	45.0	3.91, 3.40	71 Gln	121.9	7.73	58.7	3.27
29 Phe	121.7	7.37	57.2	4.73	72 Lys	121.1	7.51	58.4	3.88
30 Thr	121.1	10.98	63.9	4.18	73 Ala	123.0	8.31	53.7	1.92
31 Ser	122.3	9.15	61.1	4.42	74 Val	117.9	7.25	67.3	3.26
32 Glu	123.7	7.87	56.0	4.45	75 Glu	118.7	8.27	60.0	3.85
33 Ile	131.0	9.10	60.3	5.12	76 His	120.1	8.24	59.7	4.24
34 Thr	127.4	9.72	61.2	4.94	77 Leu	121.2	7.99	57.5	4.04
35 Val	128.4	9.29	60.8	4.86	78 Val	122.0	8.96	66.8	3.59
36 Thr	124.5	8.94	60.8	5.48	79 Lys	122.6	7.24	59.3	4.02
37 Ser	121.3	9.11	56.7	4.79	80 Leu	120.5	7.73	57.5	4.05
38 Asn	129.7	9.82	54.2	4.41	81 Met	118.4	8.09	58.2	4.06
39 Gly	105.4	8.63	45.3	4.13, 3.58	82 Ala	120.9	7.83	53.6	4.30
40 Lys	124.9	7.95	54.8	4.69	83 Glu	118.3	7.78	55.7	4.42
41 Ser	118.4	8.43	56.9	5.83	84 Leu	123.8	7.24	55.8	4.27
42 Ala	125.8	9.14	50.7	4.59	85 Glu	127.7	7.87	57.4	4.27
43 Ser	116.2	8.20	57.0	4.83					

^a $^{13}\text{C}\alpha$ and H α chemical shifts obtained from both the HNCA experiment and ^{13}C -NOESY-HMQC experiment.

one corresponding to the intra-residue $^{13}\text{C}\alpha$ resonance, the other to the $^{13}\text{C}\alpha$ resonance of the preceding residue. These ^{13}C frequencies linking neighbouring residues are connected by bars to show the agreement with the proposed sequential assignment. The resulting ^{13}C chemical shifts of all C α atoms are collected in Table 1. The inter-residue J connectivities enabled us to obtain the $^{13}\text{C}\alpha$ chemical shifts for Met1, Pro11, Pro18, His15 and Asn38.

DISCUSSION

The availability of isotope-enriched HPr and instrumentation to perform heteronuclear 3D NMR experiments enabled us to correct many of the published ^1H resonance assignments for this protein. Almost all discrepancies are in the

helical regions, leaving the published antiparallel β -sheet topology almost completely intact. The proposed irregularity in the region around residue 63 which Klevit and Waygood (1986) attributed to a β -bulge can now be ascribed to an error in the assignment of the H α resonance of this residue. There is no evidence in our spectrum for a β -bulge between residues 35 and 63; Wittekind et al. (1990) also did not observe a β -bulge between residues 35 and 63 in the β -sheet of *Bacillus subtilis* HPr.

Bax and coworkers observed a two-bond connectivity between the ^{15}N and $^{13}\text{C}\alpha$ of the preceding residue in the HNCA spectra of calmodulin for approximately 50% of the residues (Ikura et al., 1990a; Kay et al., 1990). Following their suggestion we used a shorter (22 ms) delay δ , in which the J couplings between ^{15}N and ^{13}C spins evolve. As a result, in our exper-

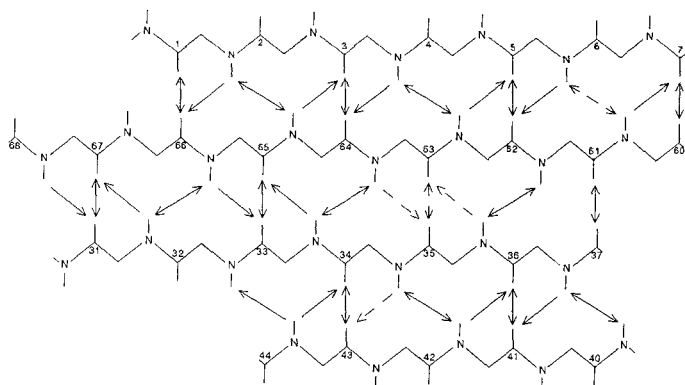


Fig. 8. The four-stranded anti-parallel β -sheet of HPr. All cross-strand NOE involving amide protons were obtained from the ^{15}N -NOESY-HMQC spectrum described above, while the $\text{H}\alpha$ - $\text{H}\alpha$ connectivities were obtained from a ^{13}C -NOESY-HMQC experiment, which will be described in a following paper. Solid arrows indicate unambiguous contacts; dotted arrows represent cases where the two protons have the same chemical shift in case of HN-HN or $\text{H}\alpha$ - $\text{H}\alpha$ contacts or where the inter-residue NOE overlaps with an intra-residue or a sequential NOE in the case of cross-strand HN- $\text{H}\alpha$ contacts.

iment this two-bond connectivity is observed for every residue leading to the complete agreement with the sequential assignment obtained from the NOESY-HMQC and TOCSY-HMQC experiments on ^{15}N -enriched HPr. This also resulted in a complete set of $^{13}\text{C}\alpha$ chemical shifts for all residues including Met1, and Pro11 and Pro18. In addition, we observed connectivities for the side chains of Gln21, Leu50, Leu77, His76, Val78, Lys79, Met81 and Glu83, all positioned in helical regions. These correlations probably result from two-bond $^2J_{\text{N,C}\beta}$ couplings and are relatively weak.

Work is in progress on ^{13}C , ^{15}N -enriched HPr to complete the ^1H resonance assignments and to identify as many NOE as possible in order to determine the 3D structure of HPr in solution.

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